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Endotoxins, Algae and 'Limulus' Amoebocyte Lysate Test in Drinking Water

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16. ABSTRACT

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ENDOTOXINS, ALGAE AND *LIMULUS* AMOEBOCYTE LYSATE TEST IN DRINKING WATER

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Abstract—Field and laboratory studies were conducted to determine the distribution of algae and bacteria, and investigate sources of endotoxins (lipopolysaccharides) in drinking water. The field survey was performed on five drinking water systems located in Allegheny County, Pennsylvania during the spring and summer of 1978. The highest concentrations of phytoplankton were found in uncovered finished water reservoirs. The major source of "endotoxic" response as measured by the *Limulus* amoebocyte lysate (LAL) gelation test was a non-specific reaction caused by algae. This was documented by a highly positive correlation of phytoplankton concentrations occurring in the reservoirs with respective LAL titers. *Chlorella vulgaris* (Chlorophyta) was the most common alga, whereas *Schizothrix calcicola* was the most dominant Cyanobacterium found in the five water systems. LAL gelation test with *C. vulgaris* grown in the laboratory verified the phenomenon observed on samples collected in the field and indicated a non-specific reaction, whereas *S. calcicola* cultures under identical conditions produced a specific response. Alkali and lysozyme treatments were successful in distinguishing specific and non-specific LAL reactions. These two techniques in conjunction with LAL test are recommended for drinking water quality assessment.

INTRODUCTION

The recent isolation of lipopolysaccharides from Cyanobacteria (Cyanophyceae or blue-green algae: Buchanan & Gibbons, 1974) suggests that in addition to Gram-negative organisms, blue-green bacteria may contaminate drinking water with these macromolecular substances (Buttke & Ingram, 1975; Keleti *et al.*, 1979; Mikheyskaya *et al.*, 1977; Weckesser *et al.*, 1974; Weise *et al.*, 1970). Lipopolysaccharides (LPS = endotoxins) are constituents of the outer cell wall of heterotrophic Gram-negative bacteria and Cyanobacteria. In both groups LPS is composed of three regions; an O-specific polysaccharide consisting of repeating oligosaccharide units which differ among species, a basal core oligosaccharide, and a hydrophobic lipid part termed lipid A. LPS affects biological systems and may elicit several mammalian physiopathological responses. The most widely studied reactions in man are endotoxemia and pyrogenicity, the latter being characterized by a biphasic fever. Other biological and physiological responses such as leucopenia, leucocytosis, shock, Schwartzman phenomenon, depression of blood pressure, induction of interferon production, dissolution of cancerous tumors and *Limulus* amoebocyte lysate (LAL) gelation have also been reported.

The occurrence of LPS in drinking water has not been intensively stud[illegible] controversy. However, numerous unexplained cases of endotoxemia that have occurred in hospitals among debilitated and immunosuppressed patients or infants are suspected to have originated from the presence of LPS in milk or water that was consumed by the victims shortly before the affliction developed.

DiLuzio & Friedman (1973) studied the distribution of LPS in different drinking waters in the United States and Mexico. Interestingly enough, the highest value they recorded (800 ng ml^{-1}) was detected in tap water of Mexico City. This high LPS level does not seem to affect the indigenous population but may cause "traveller's diarrhea." Similarly, LPS may be responsible for diarrhea in weaned infants who can be characterized as "newcomers" to the environment. The presence of LPS in drinking water and the effect of injected LPS have been observed by Hindman *et al.* (1975) while studying an epidemic of pyrogenic reactions among kidney dialysis patients. This epidemic coincided with the algal population maximum in the raw water.

The ingestion of LPS produced by algal and/or bacterial blooms in treated water stored in uncovered reservoirs may be responsible for some waterborne disease outbreaks presently classified as "of unknown etiology." For example, the presence of high concentrations of LPS accompanied by high concentrations of *Schizothrix calcicola* (400,000 cells ml^{-1}) in the Sewickley, Pennsylvania water system shortly after a

massive outbreak of waterborne gastroenteritis of unknown origin indicated a possible relationship between the consumption of endotoxins and their harmful effects (Lippy & Erb, 1976).

Evans *et al.* (1978) utilized LAL to measure the endotoxin content in stream water. They found that bound LPS (not released from cells) is a reliable measure of endotoxin associated with bacteria. Watson *et al.* (1977) have shown that the LPS content of sea water is proportional to the biomass of Gram-negative bacteria. The studies conducted by DiLuzio & Friedman (1973), Jorgensen *et al.* (1976) and Jorgensen (1979) have demonstrated the feasibility of using the LAL test for the quantification of endotoxin levels in drinking water. All three publications have shown elevated LPS levels in drinking water originating from surface sources while lower concentrations were recorded from systems treating groundwater. In spite of this effort, no attempt was ever made to test the LAL assay for non-specific reactions in water samples nor were the sources of LPS other than heterotrophic bacteria explored and discussed.

This paper examines the relationship between various organisms in drinking water and LAL gelation titer, and analyzes the non-specific reactions of the LAL assay.

MATERIALS AND METHODS

Selection of sites

Five water systems in Allegheny County (Pittsburgh, Pennsylvania) were selected for examination. Each of the systems uses uncovered reservoirs for storage of finished water:

(a) Dixmont State Hospital (Dx)
(b) City of McKeesport (McK)
(c) City of Pittsburgh (Pg)
(d) Borough of Sewickley (S)
(e) Borough of Springdale (Sp)

Table 1 contains basic technical data and brief description of the water systems. Sampling was conducted from June 12 through September 28, 1978. A total of 227 sets of samples were collected and analyzed on a weekly basis from each of the following locations in the water systems: (1) treatment plant effluent, (2) open reservoir, (3) distribution system.

Finished water was sampled at water treatment plants; open finished reservoirs at the edge of the reservoir at the farthest point from the inlet, and the distribution systems at selected points. All the samples were placed on ice for the trip back to the laboratory.

Chemical and physical analyses

The water temperature was measured in the field and all the samples were analyzed for chlorine concentration, pH, and total hardness. Determinations of free and total chlorine were performed in the field immediately upon collecting the sample using the Taylor Chlorine Field Kit® (DPD), while pH was measured in the laboratory using an ORION® pH meter equipped with a combined Fisher® electrode. The sampling procedure and determination of total hardness was that described in *Standard Methods for the Examination of Water and Wastewater*, 14th Edition (1975).

Biological analyses

Bacteriological samples were analyzed for Standard Plate Count (SPC) bacteria according to Standard Methods (1975) in duplicates; the two results were averaged in order to obtain a representative value. For the bacteria identification all the SPC colonies were characterized according to their apparent morphology, size, color and location (depth). All colony types were tested using the standard Gram staining. Samples for phytoplankton analysis were collected in 1 l bottles and preserved with Lugol's solution. After a sedimentation period of several days (minimum of 72 h) the samples were concentrated to 50 ml by careful siphoning of the supernatant. One to three aliquots of the concentrate were then quantitatively analyzed in a Palmer–Maloney nannoplankton cell. The resulting algae concentrations for the three aliquots were averaged and rounded to the nearest whole number. Individual cells were enumerated in those forms which are multi-cellular or colonial.

LAL gelation test

The LAL gelation assay was used for detection and quantitative evaluation of LPS concentrations. Samples for analyses were collected in sterile, pyrogen-free test tubes (Falcon) and were immediately placed in an ice chest for delivery to the laboratory where all samples were stored at 4°C until assayed. Analyses were conducted within one week of collection with freeze-dried LAL (Pyrotest®, Difco Laboratories) capable of detecting as little as 2.5 ng ml^{-1} of LPS. Samples were adjusted to pH between 6–8 using pyrogen-free 0.1 N hydrochloric acid or 0.1 N sodium hydroxide (Sigma Chemical Company, St. Louis, Missouri). In all pH measurements portions of the sample were decanted into 10 ml beakers for analysis.

The LAL assay procedure was modified after that of Jorgensen *et al.* (1976). Water samples and the lysate were equilibrated at room temperature prior to actual assay. All tests were conducted using sterile, pyrogen-free, 10 × 75 mm test tubes. The assays were performed by adding 0.1 ml of the reconstituted lysate before the addition of 0.1 ml of the water sample. Contents of the tubes were thoroughly agitated using a Vortex mixer. The tubes were covered with aluminium foil and incubated for 60 min at 37°C in a water bath. They were not disturbed during the entire incubation period. In order to determine the activity of each batch of the lysate, serial dilutions of the Difco positive control containing 2.5 ng ml^{-1} of *Escherichia coli* endotoxin were used. In addition, pyrogen-free Difco Pyrotrol®, and sterilized Travenol® for injection (additionally autoclaved for 1 h) were utilized as negative controls to provide information on possible pyrogen contamination of the dilution water. Sterile, pyrogen-free technique was practiced throughout the procedure.

After the 1 h incubation period the tubes were carefully inverted in order to determine presence of gelation. Responses classified only as strong gelation (solid clot) or evident gelation (clot breaks upon inversion) were interpreted as positive reactions.

Concentrations of LPS in the water samples were determined by performing serial exploratory 10-fold dilutions of the original sample with pyrogen-free water (autoclaved Travenol® for injection). In order to increase the sensitivity, additional two-fold dilution of the highest concentration displaying positive reaction were performed. For example, if the undiluted samples indicated a positive response then the final, two-fold dilutions were prepared between the undiluted sample and 1:10 dilution. The LPS concentration of each water sample tested was calculated using the established sensitivity of the lysate multiplied by the reciprocal of the greatest dilution factor which gelled the lysate.

Table 1. Summary of engineering characterization for the five water systems

System	Daily output	Population	Chemicals	Reservoir

Table 1. Summary of engineering characterization for the five water systems

System	Source	Daily output (m^3 day^{-1})	Population served	Treatment*	Chemicals added	Reservoir storage (m^3)	Remarks
Dixmont	Ohio R.	908	950	C, S, RSF, Po Cl_2	Chlorine, Alum, Soda Ash, Lime Act. Carbon	2650	No Po Cl_2 after storage
McKeesport	Youghiogheny River	37,854	59,000	C, S, ACF, Po Cl_2	Alum, Ferric Sulfate, Lime, Act. Carbon Chlorine	28,387	Boostes chlorination in reservoir
Pittsburgh	Allegheny R.	246,025	450,000	Pre Cl_2, C, S, RSF, Po Cl_2	Alum, Lime, Soda Ash, Act. Carbon, Potassium Permanganate, Chlorine Dioxide, Chlorine Chlorine	671,838	Po Cl_2 after reservoir
Sewickley	Groundwater	6814	8000	Pre Cl_2, Soft, RSF, Po Cl_2		Res. No. 3 6738 Res. No. 4 3785	Po Cl_2 after Res. No. 3
Springdale	Groundwater (well)	2233	6500	Soft, pH, Po Cl_2	Caustic Soda Chlorine	1892	No Po Cl_2 after storage, reservoir used at night. Inlet and outlet through same orifice

* C = Coagulation, S = Sedimentation, RSF = Rapid Sand Filtration, ACF = Anthracite Coal Filter, Pre Cl_2 = Pre Chlorination, Po Cl_2 = Post Chlorination, Soft = Softening, pH = pH Adjustment.

Algal cultures

Chlorella vulgaris (Chlorophyta) was isolated from Pittsburgh drinking water and *Schizothrix calcicola* (Cyanobacteria) from Mosquito Creek Reservoir (Ohio). Isolation of *S. calcicola* from drinking water was unsuccessful due to low densities present in the water supplies at the beginning of the experiment. For LAL testing, both species were mass cultured in 9 l. clear Pyrex glass bottles using Allen's medium, kept at 24°C, aerated with compressed air at a rate of 100–250 ml min^{-1}, and illuminated by "white" fluorescent light adjusted to a 16-h cycle. Each bottle was aerated prior to inoculation with 200 ml of pure starter culture.

The number and biomass of bacteria occurring in algae were determined by plating serial dilutions of algal cultures on Trypticase Soy Agar in duplicates. The plates were incubated for 72 h at 20° and 35°C. The average number of bacteria per ml was established by counting the colonies growing on the plates. The biomass was calculated utilizing *Salmonella typhimurium* as a standard. A series of dilutions in physiological saline containing this organism was plated on Trypticase Soy Agar medium and the number of bacteria present in the culture was obtained. A known aliquot of the same culture was lyophilized and the dry weight of bacteria was established. Based on this standard, the average count of bacteria in *S. calcicola* and *C. vulgaris* cultures constituted only about 0.04% of the total biomass. This contamination by heterotrophic bacteria was found to be negligible because it was caused not only by Gram-negative bacteria (containing LPS) but also by Gram-positive organisms. Furthermore, LPS isolated by Keleti *et al.* (1979) from identical *S. calcicola* cultures were characterized by the absence of KDO and heptose, common components of LPS derived from Gram-negative, heterotrophic bacteria.

Lysozyme and alkali treatment

The alkali and lysozyme treatment was used to differentiate between specific and non-specific reaction of LAL test. Alkali hydrolysis destroys LPS and lysozyme breaks down peptidoglycans into inactive compounds. Samples of *C. vulgaris* and *S. calcicola* were treated with three times crystallized egg white lysozyme (100 μg mg^{-1} at pH 6.3, in 0.15 N saline kept at 37°C for 18 h) according to Wildfeuer *et al.* (1975).

Alkali treatment was accomplished using the technique described by Suzuki *et al.* (1977). The material was suspended in pyrogen free 0.1 N sodium hydroxide solution which was kept in a sealed tube in a water bath at 70°C for 4 h. After neutralization with pyrogen free hydrochloric acid the samples were subjected to the LAL gelation test.

Statistical analysis

Linear regression analyses were performed comparing LPS concentrations with SPC bacteria and total phytoplankton. The LPS concentrations and the other variables were transformed into logarithmic values in an attempt to find the best fitting linear relationships. Using these data Pearson's correlation coefficients and significance levels were computed. The 0.05 level of significance was designated as the criterion for rejection.

RESULTS

The results of chemical analyses of drinking water samples presented in Table 2 indicate a variation in

Table 2. The arithmetic means and standard deviations of temperature, pH, hardness, free chlorine, and total chlorine values at each sampling location in this study

Sampling location	N	Temperature (°C)		pH		Hardness (mg/l)		Free chlorine (mg/l)		Total chlorine (mg/l)	
		$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s	$\bar{x}$	s
Springdale finished waters	17	15.1	2.4	7.74	0.20	18	17.0	0.38	0.14	0.52	0.15
Springdale reservoir	15	20.2	3.2	7.99	0.28	25	15.0	0.05	0.09	0.11	0.16
Springdale distribution	17	22.1	2.4	7.68	0.10	20	16.7	0.36	0.73	0.46	0.72
McKeesport finished waters	17	22.5	2.3	7.86	0.26	124	17.5	0.86	0.60	0.96	0.60
McKeesport reservoir	17	22.8	2.0	7.61	0.22	133	20.0	0.06	0.11	0.09	0.11
McKeesport distribution	16	22.8	2.3	7.94	0.26	129	15.8	0.67	0.56	0.73	0.57
Pittsburgh finished waters	12	24.9	1.5	7.55	0.33	163	11.2	0.29	0.12	0.31	0.13
Pittsburgh reservoir	12	24.7	2.0	7.70	0.20	168	13.0	0.28	0.86	0.28	0.86
Pittsburgh distribution	9	25.3	1.9	7.64	0.14	169	10.2	0.09	0.12	0.13	0.14
Dixmont finished waters	6	23.1	2.4	7.35	0.48	149	17.4	1.04	1.15	1.16	1.09
Dixmont reservoir	15	23.6	1.1	7.65	0.35	142	17.1	0	0	0.02	0.42
Dixmont distribution	15	24.8	1.8	7.57	0.45	139	17.4	0.04	0.09	0.07	0.10
Sewickley finished waters	15	16.2	1.4	7.76	0.37	129	32.0	0.97	0.78	1.52	1.03
Sewickley Reservoir No. 3 (open)	6	16.5	0.5	7.79	0.20	122	9.5	0.08	0.08	0.16	0.11
Sewickley reservoir No. 3 (covered)	8	19.7	1.5	7.63	0.30	128	11.0	0.27	0.19	0.41	0.24
Sewickley reservoir No. 4 (open)	4	23.3	0.5	8.30	0.20	97	8.1	0	0	0	0
Sewickley reservoir No. 4 (covered)	11	21.2	1.9	7.73	0.14	126	6.0	0.09	0.14	0.09	0.14
Sewickley low pressure distribution system	14	19.5	0.9	7.68	0.20	122	6.0	0.13	0.17	0.18	0.18
Sewickley high pressure distribution system	15	21.3	1.0	7.70	0.26	125	11.4	0.06	0.09	0.09	0.13

$\bar{x}$ = arithmetic mean.
s = standard deviation.
N = number of samples.

several parameters. The highest pH values were usually found in the reservoirs, whereas slightly lower values were recorded in the treatment plant effluents and distribution systems. Hardness concentrations were lowest in the Springdale system. In most of the open finished water reservoirs the chlorine (free and total) was low or even completely absent (Dixmont and Sewickley No. 4 reservoirs). The low chlorine content present in the reservoirs subsequently affects chlorine concentrations in the distribution systems supplied by the respective reservoirs unless additional booster chlorination is provided.

The geometric means of phytoplankton, bacteria and LPS in the treatment plant effluents, storage reservoirs and distribution systems are shown on Table 3. The greatest total LPS levels were found in the reservoirs with high concentrations of algae and SPC bacteria. The highest LPS values among reservoirs were recorded in small impoundments (Sewickley No. 4 and Springdale reservoirs). McKeesport reservoir samples contained almost identical LPS levels when compared with the treatment plant effluent. Sewickley reservoir No. 3 displayed very low LPS concentrations and low algal counts. In the distribution systems all biological constituents were usually present in higher concentrations than in the treatment plant effluents but were lower than the values obtained from open reservoirs.

The results of bacteriological analysis indicated that on the average, 53% of the SPC colonies were formed by gram-negative organisms. The most common group were *Flavobacteria* with occasional occurrence of *Pseudomonas* and *Serratia*. The SPC bacteria were found at elevated levels in uncovered reservoirs, namely Dixmont and Sewickley No. 4. These elevated levels may have contributed to increased endotoxin concentrations in these two reservoirs. However, the results of regression analysis of the reservoir samples (Fig. 1) indicated an insignificant relationship between SPC bacteria and LPS at the 95% confidence level ($r = 0.21089$, $p = 0.06934$). Conversely, the correlation between total phytoplankton and LPS (Fig. 2) was found to be highly significant ($r = 0.5963$, $p < 0.0001$). No relation was observed between the phytoplankton and SPC bacteria counts.

Figure 3 shows geometric means and confidence limits of total phytoplankton LPS concentrations as measured in open drinking water reservoirs. As can be seen there is an obvious correlation between total phytoplankton and LPS concentrations. The greatest values of both constituents occurred in Sewickley No. 4 and Springdale reservoirs, while lowest concentrations were recorded in Sewickley No. 3 and McKeesport water storage facilities. When comparing SPC bacteria and LPS concentrations (Fig. 4), the relationship is not as clear as that demonstrated for phytoplankton and LPS. This is evidenced by low geometric LPS means which corresponded to elevated SPC bacteria (Sewickley) while higher LPS values did not result in increased bacterial counts (Springdale).

A brown-pigmented strain of *S. calcicola* was found to be the most common Cyanobacterium occurring in the Pittsburgh area uncovered finished water reservoirs (Table 4). *S. calcicola* represented 74.5 and 18% of total phytoplankton counts in the Pittsburgh and McKeesport areas, respectively. *S. calcicola* also occurred in 89% of samples and constituted 10% of total phytoplankton population in the Pittsburgh distribution system. The same species was encountered in low numbers in samples from Sewickley low pressure system (in 80% of samples—see Table 4).

Chlorella vulgaris, a green alga, constituted from 22.8 to 99.7% of the total phytoplankton organisms counted in treated drinking water stored in open reservoirs.

In order to prevent further contamination and outbreaks similar to that which occurred in August 1975, Sewickley Reservoir Nos. 3 and 4 were completely covered with permanent plastic covers, four weeks and six weeks respectively after our sampling program had been initiated. This action had a major effect on all contaminants in the reservoirs and resulted in substantial reduction of bacteria and algae. However, in spite of the cover, algae were able to survive in low numbers for a considerable period (Table 3).

Table 5 shows the gelation of LAL reacting with various cell concentrations of *C. vulgaris* and *S. calcicola* cultures using intact as well as lysozyme and alkali treated samples. Both species of untreated organisms caused LAL gelation up to 10^5 cells ml^{-1}. The treatment with 0.1 N NaOH destroyed the LAL gelling potency of *S. calcicola* while the same alkali application had no effect on *C. vulgaris*. On the other hand, the lysozyme treated *S. calcicola* retained LAL gelation capacity, while lysozyme treated *C. vulgaris* did not induce gelation. Thus, the lysozyme treatment destroyed the gelation activity of *Chlorella* suggesting non-specific *Limulus* lysate reaction. The negative results obtained from alkali treated *S. calcicola* indicated the presence of "true" LPS in this species. In support of the data, Keleti *et al.* (1979) isolated "true" LPS from *S. calcicola*.

DISCUSSION

Variations in chemical and biological parameters

Relatively low LPS, algal and bacterial concentrations found in the McKeesport reservoir can be explained by additional chlorination of the reservoir water. The low water temperature, averaging only 16.5°C, combined with chlorine was most probably responsible for the reduction of biological constituents in the Sewickley reservoir No. 3. The increase in bacteria, algae and LPS levels in distribution systems could be caused by decomposition of algae and bacteria originating in open reservoirs and distribution systems accompanied by low chlorine levels in the mains.

Table 3. 95% confidence intervals and geometric means of phytoplankton counts, SPC bacteria and LPS

Sampling location	SPC bacteria colonies ml^{-1}			Total phytoplankton cells ml^{-1}			Cyanobacteria cells ml^{-1}			LPS ng ml^{-1}	
	N	C.I.	$\bar{x}g$	N	C.I.	$\bar{x}g$	C.I.	$\bar{x}g$	N	C.I.	$\bar{x}g$
Springdale finished waters	14	2.0–6.4	3.6	17	15–130	44	0.86–2.8	1.6	17	0.61–1.3	0.90
Springdale reservoir	14	40–1000	200	15	35000–150000	73000	2.9–34	9.9	15	4.5–22	9.9
Springdale distribution	13	7.3–92	26	17	190–2500	690	0.84–2.6	1.5	17	0.87–2.0	1.3
McKeesport finished waters	12	4.6–31	12	17	26–500	110	1.3–10	3.5	17	0.81–3.6	1.7
McKeesport reservoir	14	24–410	100	17	190–1700	560	28–290	90	17	0.72–4.3	1.8
McKeesport distribution	10	5.5–82	21	16	33–240	88	0.89–4.4	2.0	16	0.98–5.0	2.2
Pittsburgh finished waters	12	4.7–57	16	11	7.3–100	27	0.69–2.7	1.4	12	0.32–1.0	0.57
Pittsburgh reservoir	5	3.2–3400	110	12	1100–9100	3200	120–5000	770	12	2.1–9.8	4.6
Pittsburgh distribution	7	2.3–460	33	9	120–2100	490	7.0–140	31	9	0.31–2.0	0.78
Dixmont finished waters	6	0.49–14	2.7	6	6–4400	160	0.55–3.9	1.5	6	0.43–1.5	0.80
Dixmont reservoir	10	97–4400	650	15	920–1300	3400	0.95–1.2	1.1	15	2.1–10	4.6
Dixmont distribution	11	110–2600	530	15	610–9000	2300	0.76–16	3.5	15	1.8–5.4	3.1
Sewickley finished waters	13	1.3–9.1	3.4	15	7.1–140	31	0.76–3.5	1.6	15	0.37–1.5	0.73
Sewickley reservoir No. 3 (open)	4	20–21000	650	6	25–3900	310	1.8–120	14	6	0.52–3.1	1.3
Sewickley reservoir No. 3 (covered)	7	7.6–1400	100	7	1.6–25	6.3	0.75–2.0	1.2	8	0.31–1.3	0.63
Sewickley reservoir No. 4 (open)	2	160–3400	2300	4	4300–290,000	36000	130–6400	900	8	4.9–3200	130
Sewickley reservoir No. 4 (covered)	6	13–4900	260	8	3.5–130	21	0.83–1.6	1.2	11	2.7–16	6.5
Sewickley low pressure system	10	1.5–39	7.7	14	28–150	65	1.3–14	4.3	14	0.55–2.9	1.3
Sewickley high pressure system	12	5.3–150	28	15	25–690	130	1.1–12	3.7	15	1.1–6.7	2.7

N = number of samples.
C.I. = confidence interval.
$\bar{x}g$ = geometric mean.

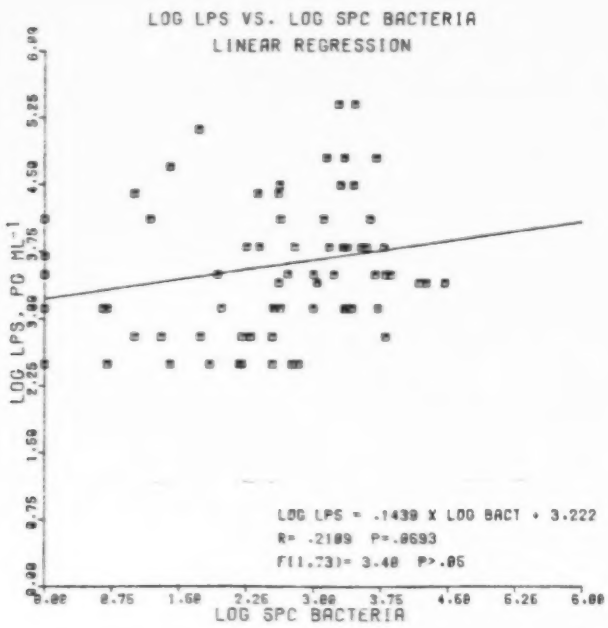
LOG LPS VS. LOG SPC BACTERIA
LINEAR REGRESSION
LOG LPS, PG ML-1
LOG SPC BACTERIA
LOG LPS = .1439 X LOG BACT + 3.222
R= .2189 P=.0693
F(1,73)= 3.48 P>.05

Fig. 1. Correlation between SPC bacteria and LPS in open, finished water reservoirs.

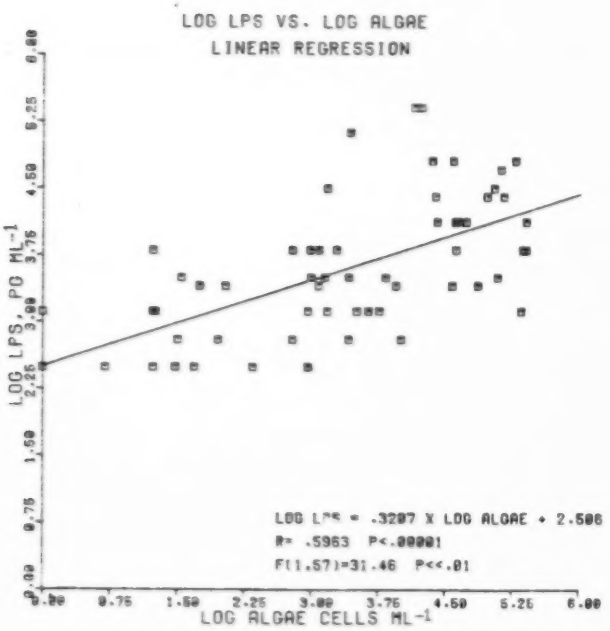
LOG LPS VS. LOG ALGAE
LINEAR REGRESSION
LOG LPS, PG ML-1
LOG ALGAE CELLS ML-1
LOG LPS = .3207 X LOG ALGAE + 2.506
R= .5963 P<.0001
F(1,57)=31.46 P<<.01

Fig. 2. Correlation between total phytoplankton and LPS in open, finished water reservoirs.

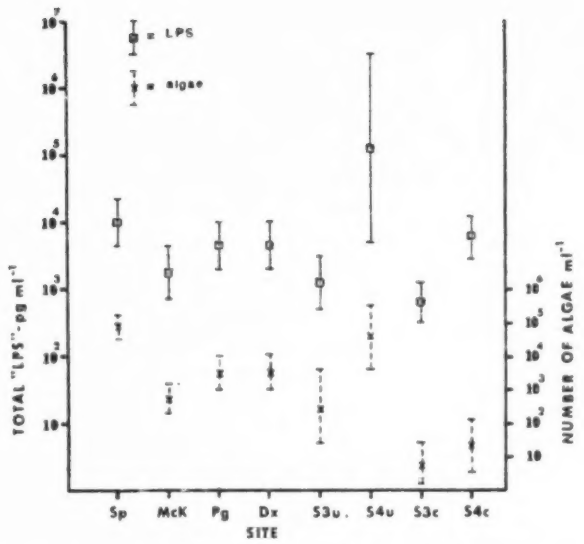

Fig. 3. LPS and Phytoplankton in open, finished water reservoirs. Vertical lines represent 95% confidence intervals with geometric means in the center. Reservoir localities: Sp = Springdale, McK = McKeesport, Pg = Pittsburgh, Dx = Dixmont, S3u = Sewickley No. 3 (uncovered), S4u = Sewickley No. 4 (uncovered), S3c = Sewickley No. 3 (covered), S4c = Sewickley No. 4 (covered).

The treatment plants which use ground water as a raw water source (Sewickley and Springdale), generally displayed low concentrations of LPS in the plant effluent. In support of this, DiLuzio & Friedman (1973) reported little or no detectable LPS levels in treated groundwater. By this criterion the Pittsburgh system appears to represent an anomaly. This treatment plant utilizes Allegheny River water; yet the plant effluent exhibited a geometric mean of only 0.57 ng ml^{-1} LPS which is substantially lower than the concentrations in the water produced at the other treatment plants. An explanation for this occurrence is that the Pittsburgh treatment plant practices prechlorination of raw water whereas Springdale and Sewickley are basically water softening plants. Thus, the degree of treatment probably affects the concen-

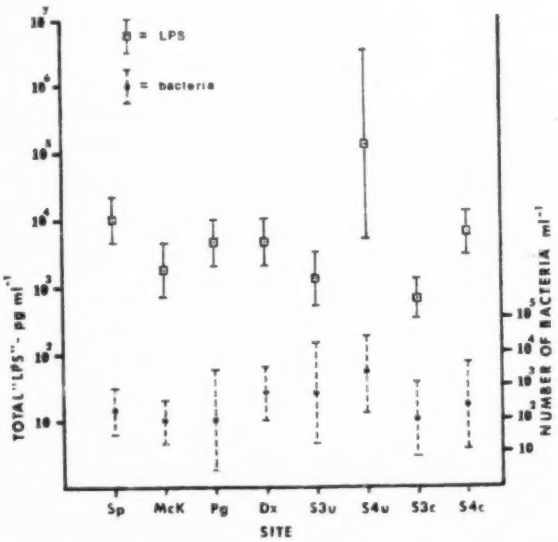

Fig. 4. LPS and SPC bacteria in open, finished water reservoirs (Legend as in Fig. 3).

Table 4. Per c

Springdale finished Reservoir
Distribution
McKeesport finished Reservoir
Distribution
Pittsburgh finished Reservoir
Distribution
Dixmont finished Reservoir
Distribution
Sewickley finished Reservoir N
(open)
Reservoir N
(covered)
Reservoir N
(open)
Reservoir N
(covered)
Low press
(open)
Low press
(covered
High press
(open)
High press
(covered

trations of total LP
than the quality of

Sources of LAL gel

Until recently, t
considered to be th
gensen *et al.* (1976)
exist between bact
also categorized th
three groups—tota
LPS is present eith
natant of the cent
determined direct
bound (in cells)
between the total
(1978) has shown
produced significa
toxin concentrati
samples collected
correlation with b
of bound endotox
metric technique.
Watson *et al.* (19
These data seen

Table 4. Per cent composition of *Chlorella, Cyanobacteria and Schizothrix calcicola* in drinking water samples

	Chlorella % of total Phytoplankton	*Cyanobacteria* % of total Phytoplankton	*S. calcicola* % of Cyanobacteria	*S. calcicola* frequency occurrence in samples (%)
Springdale				
finished	80.5	3.5	94.8	17
Reservoir	98.9	0.1	100	60
Distribution	98.6	0.1	100	12
McKeesport				
finished	96.9	1.0	99.4	41
Reservoir	81.0	18.0	100	94
Distribution	89.1	4.3	100	31
Pittsburgh				
finished	93.2	3.4	100	9
Reservoir	25.0	74.5	100	92
Distribution	85.5	10.2	99.5	89
Dixmont				
finished	98.3	0.01	0	0
Reservoir	99.7	0.001	100	7
Distribution	85.5	13.6	100	33
Sewickley				
finished	22.8	2.6	6.8	7
Reservoir No. 3				
(open)	54.3	6.0	100	83
Reservoir No. 3				
(covered)	91.8	4.1	100	14
Reservoir No. 4				
(open)	96.3	1.9	100	100
Reservoir No. 4				
(covered)	98.4	0.4	100	13
Low pressure				
(open)	49.6	31.8	100	80
Low pressure				
(covered)	77.5	19.1	100	22
High pressure				
(open)	87.1	4.7	100	50
High pressure				
(covered)	97.0	2.6	100	22

trations of total LPS in finished drinking water more than the quality of the water source.

Sources of LAL gelation

Until recently, the Gram-negative bacteria were considered to be the major source of endotoxins. Jorgensen *et al.* (1976) speculated that a correlation may exist between bacteria and endotoxins in water. He also categorized the LPS occurring in the water into three groups—total, bound and free endotoxins. Free LPS is present either in the millipore filtrate or supernatant of the centrifuged sample. Total endotoxin is determined directly in the analyzed sample and bound (in cells) LPS constitutes the difference between the total and free endotoxin. Evans *et al.* (1978) has shown that the firm clot LAL procedure produced significant correlation between total endotoxin concentrations and heterotrophic bacteria in samples collected from a small stream. An improved correlation with bacteria has been obtained by the use of bound endotoxin measured with the spectrophotometric technique. Similar results were obtained by Watson *et al.* (1977) from the marine environment.

These data seem to support the predictive capacity of the LAL assay for determination of heterotrophic bacteria in water samples. However, in our study high bacterial counts did not always occur concurrently with elevated LPS levels in water. For example, the average total SPC bacteria count in the Springdale

Table 5. *Limulus* lysate gelation of two "algal" cultures

Material (No. of cells ml^{-1})	No pretreatment	Lysozyme treated	NaOH treated
Schizothrix calcicola			
5.5×10^7	++	++	−
5.5×10^6	++	++	−
5.5×10^5	+	+	−
5.5×10^4	−	−	−
5.5×10^3	−	−	−
Chlorella vulgaris			
3.4×10^7	++	+	++
3.4×10^6	++	−	++
3.4×10^5	+	−	+
3.4×10^4	−	−	−
3.4×10^3	−	−	−

++ = firm clot.
+ = clot breaks.
− = no reaction.

reservoir (200 colonies ml^{-1}) was relatively low, but total LPS concentration was higher than in most of the other reservoirs (9.9 ng ml^{-1}). Conversely, high bacterial counts in uncovered Sewickley Reservoir No. 3 (650 colonies ml^{-1}) did not result in excessively higher LPS concentrations (1.3 ng ml^{-1}). The linear regression analysis performed on SPC bacteria and total LPS further substantiates this finding.

Another factor which must be considered when discussing endotoxin sources are algae and Cyanobacteria. The notion that some autotrophic organisms are responsible for "true" endotoxin production is supported by biochemical evidence. LPS has been isolated from the following Cyanobacteria: *Synechococcus* (*Anacystis*) *nidulans*, *Anabaena variabilis*, *Agmenellum quadruplicatum*, *Phormidium africanum*, *P. laminosum*, *P. uncinatum*, and *Schizothrix calcicola*, (Buttke & Ingram, 1975; Keleti *et al.*, 1979; Mikheyskaya *et al.*, 1977; Weckesser *et al.*, 1974; Weise *et al.*, 1970).

Cyanobacteria found in open finished water reservoirs in measurable quantity were represented by *S. calcicola*. The only time this species was present in excessive numbers in the studied reservoirs was in samples collected during and shortly after the outbreak of waterborne gastroenteritis in Sewickley, Pennsylvania in August 1975. This incident, described by Lippy & Erb (1976), was characterized as of unknown etiology, although a *S. calcicola* bloom occurred at levels as high as 400,000 cell ml^{-1} in Sewickley No. 4 reservoir.

Non-specific LAL gelation

The eukaryotic chlorophyte *Chlorella vulgaris* was the most common alga in all reservoirs. Therefore, it has been theorized that a non-specific reaction caused by *C. vulgaris* and/or some other eukaryotic algae may have been also responsible for LAL gelation. Non-specific reactions of LAL were described for a number of organic compounds by several authors. Suzuki (1977) indicated that a variety of polysaccharide derivatives such as palmitoyldextran phosphate, palmitoyldextran and dextran phosphate give a positive LAL gelation, whereas dextran itself is negative. It has been shown by Wildfeuer (1974) that gelation of LAL is induced not only by endotoxins of Gram-negative bacteria but also by peptidoglycans which may be present in various Gram-positive organisms and Cyanobacteria (Stanier & Cohen-Bazire, 1977). Rotta (1975) has also described endotoxin-like properties of peptidoglycan from Gram-positive bacteria and Fine *et al.* (1977) has shows that LAL gelation is caused by extra and intracellular lipoteichoic acid from *Streptococcus faecalis*. Serious effects of bacterial infection such as shock, disseminated intravascular coagulation and Waterhouse–Friederichsen syndrome, have also been observed in patients with Gram-positive infections (Wildfeuer *et al.*, 1975).

The peptidoglycans and possible related compounds could be effectively removed by lysozyme treatment while most of the LPS is destroyed by strong alkali (such as NaOH) hydrolysis. In spite of the fact that the lysozyme treatment effectively removes the LAL gelation capacity from *C. vulgaris* (Table 5), it has not yet been established what type of compound is responsible for the non-specific LAL reaction caused by this species. The peptidoglycans were not found in membranes of eukaryotic organisms; however, some other lysozyme-degradable substances in the gelatinous envelopes of green algae may be responsible for the positive LAL reaction.

The laboratory results also indicate that the sensitivity of LAL for detection of algae is marginal when the counts decrease below 10^5 organisms ml^{-1}. However, the analysis of field samples suggest that the algae may influence LAL relation even at levels close to 10^4 cells ml^{-1} (Tables 3 and 5). In order to determine the exact predictive value of the assay bound and free LPS (or other compounds causing gelation) determined by spectrophotometric technique should provide a more accurate representation of LPS that are contributed directly by the living organisms present in the water sample.

This study strongly suggests that *C. vulgaris* is the causative factor for LAL gelation in samples from uncovered finished water reservoirs. Therefore, the previous studies (DiLuzio & Friedman, 1973; Jorgensen, 1976) on the occurrence of LPS in drinking water may have been affected by non-specific reactions caused by various organic compounds other than "true" LPS. In spite of its deficiencies, LAL gelation is a good indicator of "general contamination" (including algae, bacteria and their by-products) of drinking water supplies. Moreover, it can provide reliable results in a relatively short period of time (approx. 1 h). Thus, LAL gelation can be a useful tool for tracing sources of contamination in waterborne disease investigations. In order to increase the accuracy and predictive value of LAL test, free and bound LPS should be measured (Jorgensen, 1976, 1979). Furthermore, the use of alkali and lysozyme treatment is recommended to distinguish between non-specific LAL reaction produced by eukaryotic algae and/or Gram-positive bacteria, and "true" endotoxins.

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